

## COMPOSITIONS AND METHODS FOR TIGR GENOTYPING ASSAYS

### FIELD OF THE INVENTION

[0001] The invention relates to the field of diagnostic assays and assays for identifying patients at risk for development of glaucoma.

### BACKGROUND OF THE INVENTION

[0002] The following description of the background of the invention is provided simply as an aid in understanding the invention and is not admitted to describe or constitute prior art to the invention.

[0003] Glaucoma is the second most common cause of blindness in the United States. It is estimated that some two million Americans have glaucoma, with half of those suffering unaware of the presence of the disease. Primary open-angle glaucoma (“POAG”) is the most common form of glaucoma, accounting for some 60 to 70% of all glaucomas.

[0004] POAG is characterized by obstruction of the normal aqueous outflow of fluids through the trabecular meshwork, canal of Schlemm, intrascleral channels, and episcleral and conjunctival veins. In open-angle glaucoma, this obstruction exists despite an angle that appears open. Generally, a patient that has not been otherwise diagnosed as having glaucoma first becomes aware of the disease due to losses in the visual field. By this point, the degree of optic nerve atrophy resulting from the disease may be quite severe, and is irreversible. Thus, early diagnosis and treatment play a key role in patient management.

[0005] Several risk factors have been identified as being related to POAG, including elevated intraocular pressure (“IOP,” 50% of patients present with an IOP of <22 mm Hg), increased age (POAG is 6x more common in persons >60 years of age), a family history of the disease (a 15x increased chance of developing glaucoma), race (African Americans are at an increased risk for more serious disease), diabetes, hypertension, myopia, and the use of corticosteroids.

[0006] While elevated IOP is a primary risk factor for development of POAG, about 1/6 of patients exhibit an IOP within the normal range. Additionally, there is currently no reliable

method for predicting which patients presenting with elevated IOP will progress to POAG. Recently, the relationship of the trabecular meshwork-inducible glucocorticoid response (“TIGR”) gene and protein have been studied for possible associations with glaucoma and related diseases. See, e.g., U.S. Patents 5,861,497, 5,916,778, 5,925,748, and 6,248,867; Morissette et al., *Nat. Genet.* 19: 319-21 (1998); Brezin et al., *Am. J. Med. Genet.* 76: 438-45 (1998); Shimizu et al., *Am. J. Ophthalmol.* 130: 165-77 (2000); Nguyen et al., *J. Biol. Chem.* 273: 6341-50 (1998); and Lindblad-Toh et al., *Nat. Genet.* 24: 381-6 (2000). Each publication and patent in the foregoing section is hereby incorporated by reference in its entirety, including all tables, figures, and claims.

## **SUMMARY OF THE INVENTION**

[0007] The present invention is drawn to methods and compositions for the screening of samples for one or more TIGR polymorphisms. The sample can be a biological sample, such as a sample from a subject. The invention can be used to determine which of a plurality of TIGR polymorphisms are present in the genome of a subject. Preferably, a plurality of different samples are assayed, each in its own individual reaction mixture, and/or several different polymorphisms in the TIGR gene are assayed in that single reaction mixture. Thus, a plurality of samples may be simultaneously assayed for several different TIGR polymorphisms in a single cycle (batch run) of the assay.

[0008] In a first aspect, the invention provides methods of testing for the presence of one or more polymorphisms of a TIGR gene, in one or more samples comprising TIGR nucleic acids, by generating a labeled nucleic acid that provides a means of identifying a particular polymorphism, thus distinguishing that polymorphism from other polymorphisms that might be present in the same gene. The particular polymorphism may be identified, for example, by determining both the length of the labeled nucleic acid and the identity of a distinctively labeled nucleotide incorporated at an end of the nucleic acid.

[0009] In preferred embodiments, these methods comprise one or more of the following steps: (a) preparing a reaction mixture that contains (i) sample nucleic acid suspected of containing a TIGR nucleic acid sequence, (ii) a nucleic acid polymerase, (iii) one or more extension primers, wherein the extension primers comprise nucleotide sequences that terminate at positions located

one nucleotide 3' from the positions of one or more preselected polymorphism(s) of interest, and (iv) one or more labeled dideoxynucleotide triphosphates, or ddNTPs; (b) incubating the reaction mixture under conditions such that extension primers that hybridize to the TIGR nucleic acids are labeled by addition of one of the ddNTPs comprising a label to the 3'-end of the detection primer, in order to generate one or more labeled oligonucleotides; and (c) detecting a signal from the labeled oligonucleotides. The presence of a specific polymorphism can be identified by the presence of a distinctive signal at a position in the sequence of the extended nucleic acid.

[0010] In certain embodiments, TIGR nucleic acid obtained from a sample is amplified to provide an amount of TIGR nucleic acid sufficient for primer extension to determine the presence or absence of one or more polymorphic forms of TIGR in the original sample. While the exemplary methods described hereinafter relate to amplification using the polymerase chain reaction ("PCR"), numerous other methods are known in the art for amplification of nucleic acids (e.g., isothermal methods, rolling circle methods, etc.). The skilled artisan will understand that these other methods may be used either in place of, or together with, PCR methods.

[0011] The phrase "TIGR nucleic acid" refers to any nucleic acid containing sequences directly associated with production of the TIGR protein, including TIGR genomic DNA, TIGR-encoding hnRNA, mature TIGR-encoding mRNA, or amplification products thereof. Any TIGR nucleic acid may be the subject of the methods described herein. In certain embodiments for example, a TIGR RNA may be reverse transcribed into DNA, and the DNA subjected to the analysis methods described hereinafter. In preferred embodiments, the methods are applied to TIGR gene sequences.

[0012] The phrases "TIGR gene sequences," "TIGR genomic DNA," and "TIGR gene" as used herein refer to the nucleic acid unit present in the genome of an animal, preferably a human, encoding the TIGR protein, and includes both the TIGR coding sequence and the upstream enhancer and promoter regions operably associated with the TIGR coding sequence in the genome.

[0013] The term "biological sample" as used herein refers to a sample obtained from a biological source, e.g., an organism, cell culture, tissue sample, etc. A biological sample can, by way of non-limiting example, consist of or comprise blood, sera, urine, feces, epidermal sample, skin

sample, cheek swab, sperm, amniotic fluid, cultured cells, bone marrow sample and/or chorionic villi. Convenient biological samples may be obtained by, for example, scraping cells from the surface of the buccal cavity.

[0014] The term “subject” as used herein refers to any eukaryotic organism. Preferred subjects are fungi, invertebrates, insects, arachnids, fish, amphibians, reptiles, birds, marsupials and mammals. A mammal can be a cat, dog, cow, pig, horse, ox, elephant, simian. Most preferred subjects are humans. A subject can be a patient, which refers to a human presenting to a medical provider for diagnosis or treatment of a disease. The term “animals” includes prenatal forms of animals, such as fetuses.

[0015] As used herein, a “plurality of samples” refers to at least two. Preferably, a plurality refers to a relatively large number of samples. A plurality of samples is from about 5 to about 500 samples, preferably about 25 to about 200 samples, most preferably from about 50 to about 200 samples. Samples that are processed in a single batch run of the method of the invention are usually prepared in plates having 24, 48, 96, 144, or 192 wells. The term “samples” includes samples per se as well as controls, standards, etc. that are included in a batch run.

[0016] A “preselected TIGR polymorphism” is a TIGR nucleic acid sequence that has been selected for testing by the methods of the invention. Examples of preselected TIGR polymorphisms include wild type TIGR, and single base polymorphisms referred to herein as MT-1 (a promoter mutation), and T377M, E423K, and N480K (all exon 3 mutations). The MT-1 mutation replaces C with G at position 906 in the promoter sequence of TIGR (Figure 2). The T377M mutation replaces C with T at position 468 in the TIGR coding sequence, leading to a substitution of Thr with Met in the TIGR protein; the N480K mutation replaces C with A at position 778 in the TIGR coding sequence, leading to a substitution of Asn with Lys in the TIGR protein; and the K423E mutation replaces A with G at position 605 in the TIGR coding sequence, leading to a substitution of Lys with Glu in the TIGR protein (Figure 1).

[0017] The assays described herein can be used to rapidly determine which of a selected group of polymorphic TIGR nucleic acid forms are present in a sample comprising TIGR nucleic acid. By “rapid” it is meant that the length of time that is taken to carry out a single batch run of the assay, from the moment a reaction mixture comprising nucleic acid is prepared to the moment a

signal can be detected, is from about 1 second to about 10, 15 or 30 seconds, about 1, 5, 10 or 30 minute(s), or 1, 3, 5, 8, 24 or 48 hour(s). When samples are from multiple subjects, the assays can be used to determine the TIGR genotype of each subject.

[0018] By “distinctively labeled”, it is meant that each type of member of a set is labeled with a label that can be distinguished from the labels used for other members of the set. For example, in a set of distinctively labeled nucleotides (e.g., dideoxy NTPs, or ddNPTs), each type of “N” (nucleotide) is labeled with a label that can be distinguished from the other types of labels. Thus, for example, if four labels designated 1, 2, 3, and 4 are used to label the four types of ddNTPs, each ddATP molecule is labeled with label “\*1”, each ddTTP molecule is labeled with label “\*2”, each ddCTP molecule is labeled with label “\*3”, and each ddGTP molecule is labeled with label “\*4”. In some aspects of the invention, the distinctive label is a fluorescent label.

[0019] The skilled artisan will understand that, if one wishes to determine if a specific genotype is present in a sample, e.g., a “T” in a position in the TIGR sequence that would be a “G” in the wild-type sequence, one need only provide a single labeled ddNTP, in this case ddTTP, with an appropriate extension primer. If the “T” polymorphism is present, the labeled ddTTP will be incorporated into the 3' end of the extension primer. In contrast, if the wild-type sequence is present, no labeled extension primer will be created. Depending on the polymorphisms selected for analysis, from one to four labeled ddNTPs may be required to perform an assay. One may also choose to include all four ddNTPs in a reaction for convenience, or so that even wild-type sequences become labeled.

[0020] As used herein, “primer extension” refers to the enzymatic extension of the three-prime (3') hydroxy group of an extension primer, which is an oligonucleotide that is paired in a duplex to a template nucleic acid. For an example of primer extension as applied to the detection of polymorphisms, see Fahy et al., Mutliplex fluorescence-based primer extension method for quantitative mutation analysis of mitochondrial DNA and its diagnostic application for Alzheimer's disease, Nucleic Acid Research 25:3102-3109, 1997. The extension reaction is catalyzed by a DNA polymerase. By “DNA Polymerase” it is meant a DNA polymerase, or a fragment thereof, that is capable of catalyzing the addition of bases to a primer sequence in a sequence-specific fashion. A DNA polymerase can be an intact DNA polymerase, a mutant

DNA polymerase, an active fragment from a DNA polymerase, such as the Klenow fragment of *E. coli* DNA polymerase, and a DNA polymerase from any species, including but not limited to thermophilic organisms.

[0021] Extension of the 3' end of the oligonucleotide generates an oligonucleotide having a length greater than the extension primer and having a sequence that is the reverse complement of the template nucleic acid. If one of the nucleotides in the added sequence is labeled, then the extended oligonucleotide becomes labeled.

[0022] Preferably, an extension primer has a nucleotide sequence that binds in a complementary fashion to a portion of a nucleic acid sequence that encodes or modulates the expression of the TIGR gene, or to the complement of such a sequence. Extension primers must be of a length sufficient to provide specific binding to the target sequence of interest. Such primers comprise an exact complement to the sequence of interest for 15 to 75 nucleotides in length, preferably 17 to 50 nucleotides in length, and more preferably from 20 to 30 nucleotides in length. The extension primer sequence has a 3' terminus that pairs with a nucleotide base that is, in the sample nucleic acid to which the primer is hybridized, 5' from the site of one or more bases in the sequence of interest that represent a polymorphism in a gene. Suitable extension primers are described herein, and may be one of the sequences set forth in SEQ ID NOS:1-4.

[0023] In addition to the sequence that ensures hybridization to the target site, an extension primer may have additional nucleotides added to the 5' end that need not participate in specific binding. Thus, such primers may extend for 15 to 75 nucleotides in length, preferably 17 to 50 nucleotides in length, and more preferably from 20 to 30 nucleotides in length, only a subset of which is an exact complement to the sequence of interest. In these embodiments, the exact complement may extend for at least 15 nucleotides, more preferably for at least 17 nucleotides, and most preferably for at least 20 nucleotides to ensure specific hybridization of the extension primer. Thus, an extension primer may contain one of the sequences set forth in SEQ ID NOS:1-4 at the 3' end, with additional nucleotides at the 5' end which may or may not be complementary to the TIGR sequence of interest.

[0024] In the following diagram of a primer extension reaction, four different ddNTPs, each distinctively labeled, are present in the reaction mixture as designated by dd(A\*1)TP,

dd(T\*2)TP, dd(C\*3)TP and dd(G\*4)TP, where \*1, \*2, \*3 and \*4 represent different labels. In the diagram, the polymorphism in the nucleic acid being tested is indicated by an underlined nucleotide, and the extension primer sequence is italicized. Only one ddNTP, ddTTP, can be added to the 3' end of the extension primer, because thymine (T) is the only base that pairs with adenosine (A). The addition of the dd(T\*2)TP to the 3' of the primer prevents any further primer extension because it is a dideoxy, chain-terminating ddNTP. Thus, the only primer that is 3' extended is labeled with label \*2. Detection of the signal from label \*2 indicates that the A polymorphism is present in the sample:

wild-type	5'-CCGGGGTGGTTGGCGAAGGCAGTCCCCTGTGCTGCC-3'
sample	5'-CCGG <u>A</u> GTGGTTGGCGAAGGCAGTCCCCTGTGCTGCC-3'
primer	3'- <i>CACCAACCGCTTCCGTCA</i> GTGGA-5'
labeled ddNTP	
dd(A <sup>*1</sup> )TP	3'-CACCAACCGCTTCCGTCA <i>GTGGA</i> -5'
dd(T <sup>*2</sup> )TP	3'-TCACCAACCGCTTCCGTCA <i>GTGGA</i> -5'
dd(C <sup>*3</sup> )TP	3'-CACCAACCGCTTCCGTCA <i>GTGGA</i> -5'
dd(G <sup>*4</sup> )TP	3'-CACCAACCGCTTCCGTCA <i>GTGGA</i> -5'

[0025] As discussed herein, an amount of nucleic acid sufficient for primer extension can, but need not be, prepared by amplification, e.g., via PCR using amplification primers. As a non-limiting example, appropriate amplification primers include, but are not limited to, those having sequences set forth in SEQ ID NOS:5-9.

[0026] For each reaction mixture, the amount of the nucleic acid sufficient for primer extension can be determined by obtaining a sample comprising nucleic acid and determining the concentration of nucleic acid therein. One skilled in the art will be able to prepare such samples to a concentration and purity necessary to practice the invention, and to estimate the amount of a specific sample that should be added to a particular reaction mixture. A failure to detect a signal in the method of the invention may signify that, among other things, an inadequate amount of nucleic acid has been added to a reaction mixture. Those skilled in the art will be able to troubleshoot failed batch runs and adjust the contents of the reaction mixtures and/or conditions of the

run accordingly. Control samples, both positive and negative, can be included in the batch runs to confirm that appropriate amounts of nucleic acid are present.

[0027] One or more of steps of the assays described herein, in any combination, are preferably performed in an automated fashion, typically using robotics, in order to provide for the processing of a large number of samples in a single batch run. Preferred forms of automation will provide for the preparation and separation of a plurality of labeled nucleic acids in small volumes. The term "small volumes" refers to volumes of liquids less than 2 ml, e.g., any volume from about 0.001 picoliters or about 0.001  $\mu$ l, to any volume about 2 ml, 500  $\mu$ l, 200  $\mu$ l, 100  $\mu$ l, 10  $\mu$ l, 1  $\mu$ l, 0.1  $\mu$ l, 0.01  $\mu$ l, or 0.001  $\mu$ l.

[0028] The set of distinctively labeled oligonucleotides generated by the methods described herein can be separated from each other so that each is mobilized in a manner that relates to each of their specific positions in the respective nucleotide sequence, and the detection of the distinctive signals generated from the distinctively labeled oligonucleotides occurs during or after the mobilization. Members of the set of distinctively labeled oligonucleotides can be separated from each other so that each is mobilized by electrophoresis. A preferred form of electrophoresis is capillary electrophoresis, but any form of electrophoresis that allows for the separation of a plurality of labeled nucleic acids in small volumes by automated or semi-automated methods and devices may be used.

[0029] In additional aspects of the present invention, polymorphic forms of TIGR assayed according to the invention can be used to diagnose subjects suffering from glaucoma or to identify subjects that are at increased risk for developing glaucoma. In preferred embodiments, such subjects may also exhibit one or more additional risk factors for glaucoma, including elevated IOP ( $\geq$  22 mm Hg, more preferably 22-30 mm Hg, most preferably 27-30 mm Hg), increased age ( $\geq$  60 years of age), a family history of glaucoma, diabetes, hypertension, myopia, and the use of corticosteroids. In certain embodiments, such subjects exhibit a normal IOP (11-21 mm Hg).

[0030] In other aspects, the results from the assays of the invention can be used to initiate or design a regimen of treatment, based on an increased risk for developing glaucoma. Such treatment may include one or more of the following: surgical or laser treatments, installation of

shunts, treatment with miotics (such as pilocarpine, carbachol, physostigmine, demecarium, and isofluorophate), treatment with carbonic anhydrase inhibitors (such as acetazolamide and methazolamide), adrenergic agonists (such as epinephrine, dipivefrine, and  $\alpha$ 2-specific agonists such as apraclonidine),  $\beta$ -blockers (such as betaxolol and metipranolol), prostaglandin analogs, and osmotic diuretics.

[0031] In yet other aspects, the present invention relates to one or more oligonucleotide molecules that are amplification primers and/or extension primers for use in the present invention. Preferably, the extension primers comprise sequences selected from the group consisting of SEQ ID NOS: 1-4, and the amplification primers comprise sequences selected from the group consisting of SEQ ID NOS: 5-8. Preferably, the oligonucleotide molecules of the present invention are purified, and most preferably substantially pure molecules.

[0032] As used herein, the term "purified" in reference to oligonucleotides does not require absolute purity. Instead, it represents an indication that the sequence is relatively more pure than in the natural environment. Such oligonucleotides may be obtained by a number of methods including, for example, laboratory synthesis, restriction enzyme digestion or PCR. A "purified" oligonucleotide is preferably at least 10% pure. A "substantially purified" oligonucleotide is preferably at least 50% pure, more preferably at least 75% pure, and most preferably at least 95% pure.

[0033] In further aspects, the present invention also relates to kits for performing the methods described herein. Preferably, such kits contain one or more extension primers in an amount sufficient to perform at least one assay for determining the presence or absence of a particular polymorphic form of TIGR in a sample. More preferably, such kits contain extension primers in an amount sufficient to perform at least one assay for determining the presence or absence of at least two, and most preferably at least four, different polymorphic forms of TIGR in a sample. Preferably, the extension primers comprise sequences selected from the group consisting of SEQ ID NOS: 1-4. In certain embodiments, the kits also contain amplification primers in an amount sufficient to perform a PCR amplification of the polymorphism region(s) of interest in the assay. Preferably, the amplification primers have sequences selected from the group consisting of SEQ ID NOS: 5-8. In certain other embodiments, the kits may also contain an instruction manual

providing instructions for use of the extension probes, and amplification probes if present in the kit.

[0034] The summary of the invention described above is non-limiting and other features and advantages of the invention will be apparent from the following detailed description of the invention, and from the claims.

## **BRIEF DESCRIPTION OF THE FIGURES**

[0035] Figure 1 represents the sequence of TIGR exon 3 (SEQ ID NO.: 9).

[0036] Figure 2 represents the sequence of TIGR promoter (SEQ ID NO.: 10).

## **DETAILED DESCRIPTION OF THE INVENTION**

[0037] The invention is drawn to assays that are predictive and/or diagnostic for glaucoma. In particular, the invention provides methods and compositions for determining the presence and sequence of variant alleles of genes encoding TIGR.

### **TIGR Polymorphisms**

[0038] In a normal diploid eukaryote, each gene has 2 loci, i.e., 1 gene copy at the same locus (position) on each of 2 matched chromosomes. Different versions of a gene can occur at any locus, and these versions are called alleles. Each allele may be the wild-type (normal) allele or an allelic variant. Thus, two different versions of a TIGR gene will be present in any particular subject.

[0039] By "allelic variant" it is meant a variation in a nucleotide sequence, such as a single nucleotide polymorphism (SNP) or any other variant nucleic acid sequence or structure (e.g., duplications, deletions, inversions, insertions, translocations, etc.) in a gene encoding a gene that alters the activity and/or expression of the gene. Allelic variants and/or over- or under-express the polypeptide encoded by the gene, and/or express proteins altered activities by virtue of having amino acid sequences that vary from wildtype sequences.

[0040] Often, more than one allelic variant exists and persists in a population of individuals. By “exist and persist” it is meant that the frequency of incidence of a rare allele(s) is greater than can be explained by recurrent mutation alone (i.e., typically greater than 1%). However, the frequency of any variant allele may vary over time due to such factors as genetic drift and the like. When 2 or more different alleles of a gene are present in a population, the gene or the protein it encodes is said to be polymorphic. As used herein, a “polymorphism” refers to a specific form of a gene or protein.

[0041] Various forms of the TIGR gene can signal varying risks of developing POAG, and/or varying severity of the disease. For example, the MT-1 mutation can be associated with greater visual field loss, elevated IOP, and refractoriness to conventional medical therapy compared to POAG patients not exhibiting this polymorphism. In the case of the T377M mutation, reported glaucoma rates for carriers are 100% by the age of 44. Similarly, for the K423E polymorphism, reported glaucoma rates for carriers are 10% by age 19, 74% between age 20 and 40, and 90% at age 40 or older; and for the N480K polymorphism, 25% by age 19, 50% by age 26, 75% by age 32, and 95% by age 57. The following table indicates the prevalence of each of these mutations in the population of POAG patients:

[0042] Mutation      Prevalence

MT-1	17%
T377M	0.12
E423K	0.4
N480K	0.81

[0043] The following additional allelic variants have been described in the TIGR gene:

TYR430HIS;  
GLY357VAL;  
GLN368TER;  
PRO370LEU;  
ILE477SER;  
PRO370LEU;  
GLY367ARG;  
GLN337ARG;  
ARG46TER; and  
CYS433ARG.

## Screening for TIGR Polymorphisms

[0044] The invention can provide rapid and simultaneous screening for large numbers of samples and/or for several polymorphisms of TIGR. In an exemplary aspect, the assays of the invention are designed to screen for 4 polymorphic loci of the TIGR gene; however, the skilled artisan will understand that the methods described herein could be expanded to provide screening for numerous additional TIGR polymorphic loci.

[0045] The screening methods described herein are discussed in reference to polymerase chain reaction (“PCR”) amplification of genomic sequences. The skilled artisan will understand that numerous methods are known in the art for amplification of nucleic acids, and that these methods may be used either in place of, or together with, the disclosed PCR steps. Nucleic acid amplification methods, such as PCR, isothermal methods, rolling circle methods, etc., are well known to the skilled artisan. See, e.g., Saiki, “Amplification of Genomic DNA” in PCR Protocols, Innis et al., Eds., Academic Press, San Diego, CA 1990, pp 13-20; Wharam et al., Nucleic Acids Res. 2001 Jun 1;29(11):E54-E54; Hafner et al., Biotechniques 2001 Apr;30(4):852-6, 858, 860 passim; Zhong et al., Biotechniques 2001 Apr;30(4):852-6, 858, 860 passim.

[0046] In embodiments where RNA (e.g., TIGR hnRNA or mRNA) is to be screened, amplification can be carried out using a combined reverse transcription-polymerase chain reaction (RT-PCR) amplification, in which a single enzyme catalyzes the primer extension both from the initial genomic RNA template (i.e. reverse transcription) and from the DNA templates synthesized in the amplification process.

[0010] Nucleic acids, including the TIGR sequence of interest, may be isolated from biological samples through the use of routine methods. Various commercial nucleic acid purification kits, such as QIAGEN® BioRobot™ and QIAamp® 96 VIRUS kit are known to the skilled artisan, and used to isolate nucleic acids.

[0047] In various embodiments, TIGR coding sequences and/or upstream regulatory sequences are amplified prior to analysis by primer extension methods, preferably using PCR. Suitable

forward and reverse primer sequences should flank the polymorphism region of interest.

Exemplary primers for two regions of the TIGR gene are as follows:

[0048] Primer Name Primer Sequence

[0049] TIGRe3F 5'-gcg gtc cca aaa ggg tca gtg tat ggt gtg tgg atg cga g-3' (SEQ ID NO.: 5)  
(exon 3)

[0050] TIGRe3R 5'-gcg gtc cca aaa ggg tca gta agt tgt ccc agg caa aga g-3' (SEQ ID NO.: 6) (exon 3)

[0051] TIGRmt1F 5'-gcg gtc cca aaa ggg tca gtg cga ata gag cca taa act ca-3' (SEQ ID NO.: 7) (promoter)

[0052] TIGRmt1R 5'-gcg gtc cca aaa ggg tca gta tct ggg gaa ctc ttc tca g-3' (SEQ ID NO.: 8) (promoter)

[0053] Amplification primer sequences may comprise a 3' end that ensures hybridization to the target site, and may also include additional nucleotides added to the 5' end that need not participate in specific binding. Thus, such amplification primers may extend for 15 to 75 nucleotides in length, preferably 17 to 50 nucleotides in length, and more preferably from 20 to 30 nucleotides in length, only a subset of which is an exact complement to the target sequence of interest. In these embodiments, the exact complement may extend for at least 15 nucleotides, more preferably for at least 17 nucleotides, and most preferably for at least 20 nucleotides to ensure specific hybridization of the amplification primer.

[0054] Once amplified, the PCR products are treated, e.g., with Shrimp Alkaline Phosphatase (SAP) and Exonuclease I, to remove excess dNTPs and PCR primers. This is followed by a single nucleotide primer extension SNaPshot reaction (Lindblad-Toh et al., Large-scale discovery and genotyping of single-nucleotide polymorphisms in the mouse. *Nature Genet.* 2000 Apr;24(4):381-6). In this reaction, an oligonucleotide primer is designed to have a 3' end that is one nucleotide 5' to a specific point mutation site or to a specific sequence found at a boundary of a mutation that occurs at a larger scale (e.g., a duplication, deletion, inversion, etc.). The primer hybridizes to the PCR amplicon in the presence of fluorescently labeled ddNTPs and a

DNA polymerase. The polymerase extends the primer by one nucleotide, adding a single, labeled ddNTP to its 3' end. Each dideoxynucleotide (e.g., ddATP, ddGTP, ddCTP, ddTTP, ddUTP, etc.) is differently labeled, e.g., each is labeled with a different fluorescent colored dye. The primers are tagged with varying lengths of nonspecific polynucleotides (e.g., poly-GACT) to allow multiplex detection of 5 or more, preferably 10 or more, and most preferably 100 or more different mutations (polymorphisms) in a single reaction. Excess ddNTPs are removed from the reaction mixture by SAP treatment. The products are fluorescently labeled oligonucleotides, each one of which may be detected, for example using an automated DNA sequencer (e.g., ABI PRISM 3100 Genetic Analyzer) based on its size (determined by electrophoretic mobility) and/or its respective fluorescent label.

[0055] Suitable extension primers for several polymorphism genotypes are as follows:

[0056] Polymorphism	Primer Name	Primer Sequence
[0057] MT-1 ID NO.: 1)	SNP32	5'-cga ata gag cca taa act caa agt ggt aat aa-3' (SEQ
[0058] T377M	SNP33Δ	5'-ccg tat tct tgg ggt ggc tac a-3' (SEQ ID NO.: 2)
[0059] K423E NO.: 3)	SNP34Δ3	5'-ctc aaa cct ggg aga caa aca tcc gt-3' (SEQ ID
[0060] N480K (SEQ ID NO.: 4)	SNP35Δ	5'-gac tgc tat aag tac agc agc atg att gac tac aa-3'

## EXAMPLES

[0061] The following examples serve to illustrate the present invention. These examples are in no way intended to limit the scope of the invention.

### [0062] EXAMPLE 1: PREPARATION OF BIOLOGICAL SAMPLES

[0063] Biological samples and other specimens are obtained, stored and prepared for assay using protocols that may vary depending on the type of sample that is to be used in the assay.

[0064] 1.1 Obtaining Specimens

[0065] Any biological sample containing TIGR nucleic acids may be a suitable sample. For genomic DNA, a convenient sample can be obtained by rinsing the mouth with water to remove loose particles, followed by scraping the cheek to collect buccal epithelial cells.

[0066] **EXAMPLE 2: INSTRUMENTS AND EQUIPMENT**

[0067] The following commercially available instruments and equipment are non-limiting examples of those that may be used to practice the invention. Those skilled in the art will be able to determine other instruments and equipment that may be used in the methods of the invention.

[0068] 2.1 Pipettes

[0069] Standard pipettes are used to deliver volumes ranging from 0.5 to 100 mL. For volumes less than 1 ml, pipettors such as the P-10, P-20, P-200, P-1000 (Rainin Instruments, LLC) pipettors are used. Pipet tips are selected from Barrier Pipet Tips (Robbins Scientific); Pipet Tips, 20 $\mu$ l and 250  $\mu$ l (Beckman), and ART (aerosol resistant tips) for P-10, P-20, P-200, P-1000 (Rainin).

[0070] 2.2 Electrophoresis

[0071] Examples of apparatuses that may be useful for electrophoresis and visualization are an agarose gel electrophoresis apparatus, such as CBS Scientific horizontal mini-gel; a power supply having a constant voltage of 200V or better variable power supply for electrophoresis, such as the BioRad Model 200; photodocumentation apparatus, such as the Alpha Innotech AlphaImager or Polaroid DS34 t; and a transilluminator, e.g., a VWR Model LM-20E or equivalent.

[0072] 2.3 Centrifugation

[0073] Centrifugation is carried in BioMek 2000 or Vortex (VWR; G-560) instruments and centrifuges for spinning PCR trays (Sorvall T6000D). The 96-well-plate centrifugation system from Qiagen may also be used. Microcentrifuges such as those from Eppendorf are used with Microcentrifuge tubes (from, e.g., National Scientific, CN065S-GT).

[0074] 2.4 PCR Containers and Reaction Plates

[0075] For DNA amplification (PCR), 2 ml MicroTubes with screw caps (Sarstedt; 72.693-005) may be used. A variety of 96-well plates suitable for PCR and other manipulations can be used. In the Examples herein, ABI MicroAmp Optical 96-well Reaction Plates (P/N#N801-0560) are used with ABI 96-well Plate Septa (P/N#4315933), or Microseal 96-well PCR microplates (MJ Research, MSP-9601) are used with Microseal A sealing film for microplates (MJ Research, MSA-5001). A 96-place storage system exemplified by VWR #30128-330, is used to store plates containing samples between steps in the assay.

[0076] 2.5 PCR Cycler

[0077] A PCR cycler capable of processing 96-well plates is used in the Examples. Exemplary PCT thermal cyclers include the GeneAmp 9600 (Perkin-Elmer) or the PTC 200 (MJ Research). The MJR PTC 200 has features that are desirable regardless of which instrument is used: heating rates of up to 3°C/second, which reduce reaction times, and rapid temperature homogeneity (e.g.,  $\pm 0.4^\circ\text{C}$  within 30 seconds at 90°C). The heating block that is used may be, for example, VWR's Heat Block (VWR, 13259-007).

[0078] 2.6 Automated Laboratory Workstation

[0079] In order to process a large number of samples, a multipurpose automated or semi-automated programmable workstation is used (Meldrum, Automation for Genomics, Part One: Preparation for Sequencing, Genome Research, 10:1081-1092, 2000; Meldrum, Automation for Genomics, Part Two: Squencers, Microarrays, and Future Trends, Genome Research, 10:1288-1303, 2000). Preferred features of the workstation include the ability to rapidly and accurately pipette, dilute and dispense small volumes of liquids. The exemplary programmable workstation used herein is the BioMek® 2000 (Beckman Coulter, Inc.).

[0080] 2.7 Capillary Electrophoresis DNA Sequencer

[0081] For high throughput of PCR products, an automated capillary electrophoresis (CE) system is used in order to separate labeled DNA molecules in a size-dependent manner, so that signals corresponding to each nucleotide in a sequence are detected in a sequential fashion. For

reviews of the use of CE in DNA sequencing and polymorphism analysis, see Heller, Electrophoresis 22:629-43, 2001; Dovichi et al., Methods Mol Biol 167:225-39, 2001; Mitchelson, Methods Mol Biol 162:3-26, 2001; and Dolnik, J Biochem Biophys Methods 41:103-19, 1999. In the Examples, the ABI PRISM® 3100 Genetic Analyzer is used with an ABI PRISM 3100 capillary array, 36-cm (P/N#4315931). This provides a multi-color fluorescence-based DNA analysis system that uses capillary electrophoresis with 16 capillaries operating in parallel to separate labeled PCR products. A CE DNA sequencer/analyser that operates 96 capillaries may be preferable in assays wherein 96-well plates are used. Analyzers with the capacity to process 96 wells include the MegaBACE™ 1000 DNA Analysis System (Molecular Dynamics, Inc and Amersham Pharmacia Biotech) and the 3700 DNA Analyzer from (Perkin-Elmer Biosystems)

### [0082] EXAMPLE 3: REAGENTS

#### [0083] 3.1 Stock Reagents

[0084] The following exemplary stock reagents are used and are stable for the indicated times when stored at the indicated temperature/conditions.

[0085] 3.1.1 Agarose, SeaKem GTG (FMC 50074). Store ambient (18°C-26°C), stable for 1 year.

[0086] 3.1.2 dNTP set, ultrapure, 100 mM solution (Pharmacia 27-2035-01). Store at -10°C to -30°C, stable for 1 year.

[0087] 3.1.3 EDTA, disodium (Sigma E-5134). Store ambient (18°C-26°C), stable for 1 year.

[0088] 3.1.4 Ethidium bromide (Life Technologies 15585-011). Store ambient (18°C-26°C), stable for 1 year.

[0089] 3.1.4 Ficoll (Sigma, Cat. #F2637). Store at 18-25°C, stable for 1 year.

[0090] 3.1.5 Bromophenol Blue (Sigma, cat. #B6131). Store at 18-25°C, stable for 1 year.

[0091] 3.1.6 Xylene Cyanol (Kodak, cat. #1B72120). Store at 18-25°C, stable for 1 year.

[0092] 3.1.7 0.5 M EDTA, pH 8.0 (Amresco, cat. #E177) , Store at 18-25°C, stable for 1 year.

[0093] 3.1.8 Taq Extender PCR additive (Stratagene 600148) stored at -20°C, stable for 1 year.

[0094] 3.1.9 If a commercially available DNA extraction kit is not used, reagents for the Proteinase K or phenol-chloroform extraction method should be prepared as is known in the art.

[0095] 3.1.10 ABI 3100 POP-4 polymer (P/N4316335), stable for 1 year when stored at 2 to 10°C.

## [0096] 3.2 Stock Solutions

[0097] The following exemplary stock solutions are used and are stable for the indicated times when stored at the indicated temperature/conditions.

[0098] 3.2.1 Water, molecular biology grade (BioWhittaker 16-001Y or equivalent) stored ambient (18°C-26°C), stable for 1 year.

[0099] 3.2.2 6x Gel loading dye (no xylene cyanol)

[0100] 3.2.3 100 mM disodium EDTA pH 8.0

[0101] 3.2.4 6%-12% (w/v) Ficoll 400

[0102] 3.2.5 0.25% (w/v) bromophenol blue

[0103] 3.2.6 10X TBE buffer

[0104] 3.2.6.1 Prepare as 890 mM Tris Base, 890 mM Boric Acid, and 20 mM Disodium EDTA

[0105] 3.2.6.2 TBE buffer (Amresco 0658 or equivalent) stored ambient (18°C-26°C), stable for 1 year.

[0106] 3.2.7 ABI 10X Buffer (P/N402824), stored at 2 to 10°C, stable for 1 year.

[0107] 3.2.8 ABI Hi Di Formamide (P/N4311320). Stored at -10°C or colder, stable for 1 year or until the indicated expiration date.

[0108] 3.2.10 100X TE buffer (Sigma T-9285 or equivalent) stored ambient (18°C-26°C), stable for 1 year.

[0109] 3.2.11 ABI 5X Sequencing Buffer, PE Applied Biosystems, (P/N4305603), stored at -15°C to -25°C, stable for 1 year.

### [0110] 3.3 Kits

[0111] The following exemplary kits may be used and are stable for the indicated time when stored at the indicated temperature/conditions.

[0112] 3.3.1 ABI SNAPshot multiplex kit (P/N4323161), stored at -10 to -30°C, stable for 6 months

[0113] 3.3.2 HotStarTaq™ PCR Core Kit (Qiagen 203203 or 203205) (HotStarTaq™ enzyme, 25mMg++, M10X buffer & 5X Q Solution), stable for 1 year when stored at -10°C to -30°C.

### [0114] 3.4 Enzymes

[0115] The following exemplary enzymes may be used and are stable for the indicated time when stored at the indicated temperature/conditions.

[0116] 3.4.1 Shrimp Alkaline Phosphatase (USB Corporation, P/N70092X), stable for 6 months when stored at -10 to -30°C

[0117] 3.4.2 Exonuclease I (USB Corporation, P/N70073X), stable for 6 months when stored at -10 to -30°C.

### [0118] 3.5 Standards

[0119] The following exemplary standards may be used and are stable for the indicated time when stored at the indicated temperature/conditions.

[0120] 3.5.1 DNA ladder, 50 bp (Pharmacia Biotech 27-4005-01) stable for 1 year when stored at -20°C

[0121] 3.5.2 ABI GeneScan-120 LIZ Size Standard (P/N4322362), stable for six months when stored at 2 to 10°C.

[0122] 3.6 PCR Amplification Primers

[0123] Oligonucleotides used as PCR primers were prepared by Operon Technologies, Inc. (0.05, 0.2 or 1.0 micromole scale synthesis, no HPLC purification) and stored as 100 µM stocks at -10°C or colder, conditions under which they are stable for 1 year. Table 2 gives the sequences of PCR primers used in the Examples.

[0124] 3.7 Primer Extension Primers

[0125] Primer extension primers were prepared by Operon Technologies, Inc. (0.05, 0.2 or 1.0 micromole scale synthesis, HPLC purification): stored as 100 µM stocks at -10°C or colder, stable for 1 year. The sequences of primers used in the examples are shown in SEQ ID NOS.: 1-4.

[0126] 3.8 Working Stocks for PCR, Primer Extension, and SAP Treatment

[0127] 3.8.1 5x Primer Mix for TIGR Duplex PCR is prepared according to the following recipe and is stable for 1 year when stored at -70°C.

Primer (100 µM)	Volume	[5X]	[working]
TIGRe3F	384 µl	2 µM	0.400 µM
TIGRe3R	384 µl	2 µM	0.400 µM
TIGRmt-1F	384 µl	2 µM	0.400 µM
TIGRmt-1R	384 µl	2 µM	0.400 µM
H2O:	17664 µl		
<b>Total:</b>	<b>19200.0 µl</b>		

[0128] 3.8.1.2 TIGR PCR Master Mix is prepared according to the following recipe.

Components	Per Rxn (μL)	x3000 (μL)
10X Qiagen PCR Buffer	2.5	33000
25 mM dNTP mix	0.25	7500
5X primer mix	5.0	15000
25 mM MgCl <sub>2</sub>	1.0	3000
H <sub>2</sub> O	11.0	33000
<b>Total</b>	<b>19.75</b>	<b>59250 μL</b>

[0129] 3.8.4 SAP+ExoI Cocktail

[0130] Combine 5 μl of SAP (1 unit/μl) and 0.2 μl of Exo I (10 unit/μl) in 1x SAP buffer to a final volume of 15 μl per reaction. The SAP+ExoI Cocktail is prepared fresh before each use.

	Concentration	Volume (μl) for 120 rxns (full plate)
SAP	1 unit/μl	600
Exo I	10 unit/μl	24
10x SAP buffer	10x	240
Sterile H <sub>2</sub> O		936
<b>Total</b>		<b>1800</b>

3.8.5 Primer Extension Primer Mix is prepared according to the following recipe.

Primer	Concentration (μM)	Volume of primer added (1 rxn)	Volume of primer added (200 rxns)
SNP32 (MT-1)	100	0.005 μL	10 μL
SNP33Δ (T377M)	100	0.02 μL	40 μL

SNP34Δ3 (K423E)	100	0.02 $\mu$ L	40 $\mu$ L
SNP35Δ (N480K)	100	0.02 $\mu$ L	40 $\mu$ L
dH <sub>2</sub> O		0.935 $\mu$ L	1870 $\mu$ L
<b>Total:</b>		<b>1.0 <math>\mu</math>L</b>	<b>2000 <math>\mu</math>L</b>

[0131] The mix is prepared in 15 ml sterile conical tubes and dispensed in 1 to 1.5 ml aliquots per microcentrifuge tube and stored at -70°C or colder.

#### [0132] 3.8.6 SNaPshot Primer Extension Master Mix

[0133] Combine 2.5  $\mu$ L of ABI SNaPshot Ready Mix, 2.5  $\mu$ L of 5X sequencing buffer, 1  $\mu$ L of Primer Extension Primer Mix and 1  $\mu$ l Sterile H<sub>2</sub>O to a final volume of 7  $\mu$ l per reaction. The Mix is prepared fresh before each use, and kept on ice until used.

Reagent	Per Well	Per Plate
SnaPshot Ready Mix	2.5 $\mu$ L	280 $\mu$ L
5X sequencing buffer	2.5 $\mu$ L	280 $\mu$ L
Extension Primer Mix	1 $\mu$ L	112 $\mu$ L
DH <sub>2</sub> O	1 $\mu$ L	112 $\mu$ L
<b>Total</b>	<b>7 <math>\mu</math>L</b>	<b>784 <math>\mu</math>L</b>

#### [0134] 3.8.7 Second SAP Cocktail:

[0135] For each reaction, 1  $\mu$ l of SAP (1 unit/ $\mu$ l) and 1  $\mu$ l of water are combined to a final volume of 2  $\mu$ l. The SAP cocktail is freshly prepared before each use.

[0136] 3.8.8 Loading Mix: Ten (10)  $\mu$ l of Hi-Di Formamide and 0.5  $\mu$ l GeneScan 120 LIZ Size Standard are combined to a final volume of 10.5  $\mu$ l per sample. Lodging Mix is prepared fresh before each use.

Reagent	Per Well	Per Plate*
Hi-Di Formamide	10 $\mu$ l	1120 $\mu$ l
GeneScan 120 LIZ Size Standard	0.5 $\mu$ l	56 $\mu$ l
<b>Total</b>	<b>10.5 <math>\mu</math>l</b>	<b>1176 <math>\mu</math>l</b>

[0137] **EXAMPLE 4: PROCEDURE**

[0138] 4.1 Preparation of Sample Trays

[0139] PCR master mix is prepared as described above and is used in the reaction. The following table describes a recipe that results in a sufficient volume for a full PCR plate (sample tray; 96-wells), and allows for excessive solution to enable pipetting from a trough with an 8-channel pipettor into all PCR wells.

	1 Rxn	Cocktail x 56 (1/2 plate)	Cocktail x 112 (full plate)
Master Mix	19.75 $\mu$ L	1106 $\mu$ L	2212 $\mu$ L
HotStarTaq	0.25 $\mu$ L	14 $\mu$ L	28 $\mu$ L
Buccal Swab / Qiagen DNA*	5.0 $\mu$ L (or 2.0 Qiagen DNA + 3.0	----	----

	dH <sub>2</sub> O)		
Total	25 µL		

[0140] If Qiagen DNA is used, add 2.0 µL DNA sample + 3.0 µL H<sub>2</sub>O per reaction; if a DNA sample is extracted with the phenol/chloroform method, it should be diluted in sterile water to a concentration of 8-16 µg/ml, and add 5 µL per reaction.

#### [0141] 4.2 PCR Reactions

[0142] For automated PCR setup on the BioMek 2000 robotic workstation, the PCR tray, a box of Robbins 125 µL pipet tips, a box of 20 µL pipet tips, the Qiagen sample tray and the reagent reservoir (trough) are placed at the appropriate positions on the BioMek work surface. If the PCR or subsequent steps are set up manually, the same master mix recipe/digestion recipe is used, and the assay proceeds as described below without the BioMek, and single or multichannel pipettors and tips are used.

[0143] The master mix is added to the reagent reservoir. Eight positions at the end of the Qiagen sample tray are left open for controls. The sample tray is briefly spun down in a plate centrifuge outside of the master mix and template addition area (i.e., in a clean room). The control samples (typically, four positive and two negative controls) are placed in the appropriate positions in the sample tray.

[0144] The BioMek station first pipets 20 µl of the master mix into each 0.2 ml PCR tray wells, and then adds 5 µl specimen DNA or control. The wells are tightly sealed with PCR tube caps or Microseal A film. The sample tray is briefly (~ 5 s) vortexed and spun down for about 30 s in a plate centrifuge at 2,000-6,000g (1,600 rpm in a Sorvall T6000D centrifuge).

[0145] The cycling program (below) is started on a thermal cycler such as the MJR PTC 200. When the temperature reaches >85°C, the PCR tray is placed in the thermal cycler and its lid is sealed.

[0146] The cycling parameters are:

Step	Temperature	Time
1	95°C	15 min.
2	94°C	10 sec
3	55°C	0.50 °C / sec. Ramp
4	55°C	10 sec.
5	72°C	0.30 °C /sec. Ramp
6	72°C	15 sec.
7	94°C	0.50 °C / sec. Ramp
8	[Go to step 2 and repeat for 32 cycles*]	
9	70°C	5 min.
10	4°C	Hold

\*Typically, 32 cycles is optimal, however 31-33 cycles may be used if the PCR products from 32 cycles are less than optimal.

[0147] After PCR is complete, the products may be stored refrigerated up to one week or frozen (<-10°C) if a longer storage period is necessary, or they may be used immediately in the following procedures.

#### [0148] 4.3 First SAP and ExoI Digestion

[0149] Digestion starts by adding 5 µl of PCR product and 15 µl of the SAP+ExoI Cocktail. The plate is sealed, vortexed and spun down in the plate centrifuge. The plate is then placed in the MJR PTC 200 thermal cycler and a cycling program is run using the following parameters.

Step	Temperature	Time
1	37°C	2 hr.
2	75°C	15 min.
3	4 °C	Hold

[0150] Each step uses rapid (default) ramp to reach desired temperature. The SAP/ExoI -treated PCR products can be stored at 2-8°C until use.

#### [0151] 4.4 Primer Extension

[0152] SNaPshot Primer extension Master Mix is freshly prepared as described above, and 7  $\mu$ l of the master mix is added to 3  $\mu$ l of digestion product from Example 4.3. After addition of the SNaPshot Primer Extension Master Mix, each plate is immediately placed in the thermocycler and the “SNAPSHOT” program is immediately run.

[0153] The plate should not be allowed to sit at room temperature more than 30 seconds. The plate is sealed, vortexed and spun down in the plate centrifuge. The plate is then placed in the MJR PTC 200 thermal cycler and a cycling program is run using the following parameters.

Step	Temperature	Time
1	96°C	10 sec.
2	50°C	5 sec.
3	60 °C	30 sec.
4		Go to step 1 24 more times.
5	4 °C	Hold

[0154] Each step uses rapid (default) ramp to reach desired temperature. The reaction plates are stored at 2-8°C until use.

#### [0155] 4.5 Second SAP Digestion

[0156] 2  $\mu$ l of the SAP Cocktail is mixed with 10  $\mu$ l primer extension product from Example 4.4. The plate is sealed and vortexed, and then spun down in the plate centrifuge. The plate is placed in the MJR PTC 200 thermal cycler and a cycling program is run using the following parameters.

Step	Temperature	Time
1	37°C	1 hr.
2	75°C	15 min.
3	4 °C	Hold

[0157] Each step uses rapid (default) ramp to reach desired temperature. The digestion plate is stored at -15°C or lower until use.

[0158] 4.6 Electrophoresis on ABI 3100 Genetic Analyzer

[0159] SAP-digested samples are prepared according to Example 4.5 for loading using a BioMek 2000. The SNaPShot product is diluted 15-fold with water, and then 2 µl of the diluted product is mixed with 10.5 µl of the Loading Mix. The plate is covered with septa, vortexed and spun down in the plate centrifuge. The plate is heated at 95°C for 5 minutes, then immediately placed on ice for 3 minutes or until use. The plate is spun down in a plate centrifuge to collect condensation. The plate is then assembled and loaded onto the ABI3100 Genetic Analyzer.

[0160] The contents of the articles, patents, and patent applications, and all other documents and electronically available information mentioned or cited herein, are hereby incorporated by reference in their entirety to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference. Applicants reserve the right to physically incorporate into this application any and all materials and information from any such articles, patents, patent applications, or other physical and electronic documents.

[0161] The inventions illustratively described herein may suitably be practiced in the absence of any element or elements, limitation or limitations, not specifically disclosed herein. Thus, for example, the terms "comprising", "including," "containing", etc. shall be read expansively and without limitation. Additionally, the terms and expressions employed herein have been used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the inventions embodied therein herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention.

[0162] The invention has been described broadly and generically herein. Each of the narrower species and subgeneric groupings falling within the generic disclosure also form part of the

invention. This includes the generic description of the invention with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

[0163] Other embodiments are within the following claims. In addition, where features or aspects of the invention are described in terms of Markush groups, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group.